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Contribution of the Global Regulator Gene *gacA* to Persistence and Dissemination of *Pseudomonas fluorescens* Biocontrol Strain CHA0 Introduced into Soil Microcosms

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Structural and regulatory genes involved in the synthesis of antimicrobial metabolites are essential for the biocontrol activity of fluorescent pseudomonads and, in principle, amenable to genetic engineering for strain improvement. An eventual large-scale release of such bacteria raises the question of whether such genes also contribute to the persistence and dissemination of the bacteria in soil ecosystems. *Pseudomonas fluorescens* wild-type strain CHA0 protects plants against a variety of fungal diseases and produces several antimicrobial metabolites. The regulatory gene *gacA* globally controls antibiotic production and is crucial for disease suppression in CHA0. This gene also regulates the production of extracellular protease and phospholipase. The contribution of *gacA* to survival and vertical translocation of CHA0 in soil microcosms of increasing complexity was studied in coinoculation experiments with the wild type and a *gacA* mutant which lacks antibiotics and some exoenzymes. Both strains were marked with spontaneous resistance to rifampin. In a closed system with sterile soil, strain CHA0 and the *gacA* mutant multiplied for several weeks, whereas these strains declined exponentially in nonsterile soil of different Swiss origins. The *gacA* mutant was less persistent in nonrhizosphere raw soil than was the wild type, but no competitive disadvantage when colonizing the rhizosphere and roots of wheat was found in the particular soil type and during the period studied. Vertical translocation was assessed after strains had been applied to undisturbed, long (60-cm) or short (20-cm) soil columns, both planted with wheat. A smaller number of cells of the *gacA* mutant than of the wild type were detected in the percolated water and in different depths of the soil column. Single-strain inoculation gave similar results in all microcosms tested. We conclude that mutation in a single regulatory gene involved in antibiotic and exoenzyme synthesis can affect the survival of *P. fluorescens* more profoundly in unplanted soil than in the rhizosphere.

Certain root-colonizing *Pseudomonas* strains are capable of suppressing plant diseases caused by soilborne pathogens (13, 14, 31, 35, 37, 44, 60). At least part of their disease-suppressive capacity has been attributed to the production of antimicrobial metabolites. Genes coding for the biosynthesis and regulation of some of these metabolites have been cloned and in part sequenced (17, 22, 25, 27, 33, 41, 42, 52, 57–59). Since the genes encoding relevant traits in these bacteria are amenable to manipulation either at the level of regulation or by transfer to new hosts, it seems possible to construct biocontrol agents which achieve better protection of certain plant species than do the parental strains (17, 22, 39, 51, 59).

Protection of agricultural crops against soilborne diseases with wild-type or genetically engineered bacteria on a commercial scale requires the release of large numbers of these organisms into the environment. The potential risks associated with such release need to be assessed. Major concerns are (i) persistence, multiplication, and dissemination of introduced microorganisms with possible adverse effects on indigenous organisms and (ii) gene transfer to resident organisms (6, 45). In this context, it is also important to know which characteristics of introduced organisms are crucial for ecological competence and whether competitiveness can be changed by genetic manipulation. Structural and regulatory genes for production of antimicrobial metabolites are, as mentioned above, likely targets of genetic engineering in biocontrol strains (7, 16). Therefore, it is of interest to see whether these genes also contribute to the strains' fitness in soil and in the rhizosphere. Over experimental periods of short duration, the loss of antibiotic production has no influence on the capacity of various *Pseudomonas* strains to colonize the rhizosphere (25, 32, 52, 53). However, in long-term experiments a reduced fitness in soil and in the rhizosphere of wheat has been found for fluorescent pseudomonads defective in the production of phenazine antibiotics (40).

Contained microcosms have been proposed as tools for risk assessment prior to field release of microorganisms (4–6). Microcosms for studying the survival of introduced bacteria may consist of closed systems with unplanted soil of constant moisture (10, 62). Because of their low variability, such microcosms may be used to detect small differences between a wild type and a mutant. More-complex systems, e.g., large, undisturbed soil cores, allow vertical translocation to be determined and are more suitable to simulate field conditions (2, 4, 5, 18, 55).

In the work presented here, we used *Pseudomonas fluorescens* CHA0 as a model strain for studies on the persistence and dissemination of introduced biocontrol agents. Strain CHA0 suppresses various soilborne plant diseases (12, 13, 23) and produces the antimicrobial metabolites hydrogen cyanide, 2,4-diacetylphloroglucinol, and pyoluteorin, which contribute to the strain's suppressive capacity (26, 27, 29, 38, 39, 58, 59). A regulatory gene, *gacA*, which globally controls the synthesis of

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TABLE 1. Physical properties of sandy loam soils used in this study

Designation[a]	% (wt/wt)				pH	Reference
	Organic matter	Sand	Silt	Clay		
Morens soil MS1	2.4	46	39	15	6.2	50
Morens soil ME2	2.6	52	31	17	6.3	Unpublished
Eschikon soil	5.9	43	42	15	7.7	Unpublished

[a] Morens soil MS1 comes from the site where CHA0 originally had been isolated (49); Morens soil ME2 is from the same region; Eschikon soil comes from a site where field experiments have been conducted (12, 63).

these antimicrobial and other secondary metabolites, has been cloned and sequenced (33). The *gacA* gene is also required for extracellular protease and phospholipase (43). The *gacA* mutants are pleiotropically blocked in the synthesis of these antimicrobial metabolites and exoenzymes and show strongly reduced protection from black root rot of tobacco (33, 43). The main purpose of this study is to determine the contribution of *gacA*-regulated traits to persistence and vertical translocation of strain CHA0. Therefore, the wild type and *gacA* mutants were compared in soil microcosms of increasing complexity, ranging from closed systems with nonrhizosphere soil to undisturbed soil cores planted with wheat and simulating an agricultural soil horizon.

MATERIALS AND METHODS

Organisms and culture conditions. *P. fluorescens* (Trevisan) Migula wild-type strain CHA0 (49) and its *gacA* mutants CHA96 and CHA89 (33) were cultivated in nutrient yeast broth (46) and on King's medium B agar (KBA) (30) as described previously (28, 59). Strain CHA96 contains a *gacA'-'lacZ* translational fusion introduced into the chromosome by gene replacement; in strain CHA89 the *gacA* fragment is replaced with a kanamycin resistance cassette (33). For easy recovery from microcosms, strains were marked with a spontaneous rifampin resistance marker. The suitability of rifampin resistance as a marker for monitoring populations of introduced bacteria has already been demonstrated by other authors (20). Rifampin-resistant (Rif^r) derivatives of strains CHA0, CHA89, and CHA96 were isolated on KBA containing 100 μg of rifampin per ml, and the same antibiotic concentration was added to the media for maintaining and cultivating these strains. Since strains CHA0, CHA96, and CHA89 were marked separately, it is possible that they carry different Rif^r alleles. However, it seems unlikely that these alleles differentially affected the fitness of the bacteria. The Rif^r derivatives were compared with the parental strains, and no differences were observed in growth rates, secondary metabolite production, prototrophy, fungal inhibition on agar media, survival in sterile soil, and disease suppression. The Rif^r mutation did not revert to a Rif^s phenotype after several passages in culture media and sterile soil. Therefore, all experiments in this study were conducted with the Rif^r derivatives of strains CHA0, CHA96, and CHA89.

For use in microcosms, seeds of wheat (*Triticum aestivum* L. 'Arina') were surface disinfected in 5% (wt/vol) sodium hypochlorite for 10 min, rinsed with sterile-filtered water, and then germinated for 3 days on 0.85% water agar (Difco Laboratories, Detroit, Mich.).

Bacterial application to microcosms and growth conditions. Unless otherwise specified, soils used in this study (Table 1) were collected from the upper 20 cm of the soil profile, passed through a 5-mm-mesh screen, and stored at 15°C prior to use. For inoculation of soil or wheat seedlings, overnight bacterial cultures grown on KBA at 24°C were harvested and washed twice in sterile distilled water. The optical density (600 nm) of the bacterial suspension was measured, and the cell density was adjusted to the desired concentration. Bacteria were then applied to soil by spraying the suspension with a chromatography sprayer and compressed air. Soil was constantly mixed during application. Most experiments were conducted in parallel with coinoculation and single-strain inoculation of the wild type and the *gacA* mutant. The inocula described below were used in coinoculation experiments. In experiments with strains applied separately, double the number of cells was used in order to obtain the same total concentration of introduced bacteria. Microcosms described below were set up in a growth chamber containing 70% relative humidity with light (160 microeinsteins m^{-2} s^{-1}) for 16 h at 18°C followed by an 8-h dark period at 13°C.

Closed microcosms with sterile and nonsterile soil. Pasteurized or nonsterile soil (Table 1) was inoculated with a mixture of the wild type and a *gacA* mutant to give 5×10^6 CFU of each strain per g. Inoculated soil (400 g) was placed in 1-liter flat-bottomed flasks. For pasteurization, soil was autoclaved twice for 30 min with an interval of 1 day before use. Soil moisture was kept constant at 17% by checking changes in sample weight and adding sterile distilled water when necessary. A soil sample (about 5 g) was periodically taken from each flask for monitoring the population of introduced bacteria.

Pot microcosms with seedling inoculation. Wheat seedlings were incubated for 15 min in a bacterial suspension containing 5×10^7 CFU (each) of the wild type and the *gacA* mutant per ml and then planted into pots containing 400 ml of nonsterile soil from Eschikon (Switzerland) (Table 1). The inoculum size was determined by shaking some of the seedlings in sterile distilled water on a rotary shaker (radius, 0.6 cm) at 300 rpm for 1 h and then enumerating the bacteria in this suspension. Three times a week the pots were irrigated from above with sterile distilled water. After 3 weeks plants were fertilized once with modified Knop's nutrient solution (28). Plants were harvested after 21 and 42 days, and the bacterial populations in the rhizosphere and on the roots were quantified.

Large open microcosms with undisturbed soil cores. Large open microcosms with undisturbed soil cores consisted of a polyvinyl chloride pipe (19-cm internal diameter) containing an undisturbed deeper-layer soil core (40 cm) covered by a 20-cm layer of sieved, homogenized surface soil. The system is an adaptation of the American Society for Testing and Materials standard microcosm (56) designed to test percolation of chemicals. A diagrammatic illustration of the experimental system is given elsewhere (58). Undisturbed soil cores were obtained from the Eschikon field site (Table 1) as follows. Twenty centimeters of surface soil was removed and saved; then, all soil around the cores (of about 18-cm diameter and 40-cm length) was carefully removed. At the field site a polyvinyl chloride pipe was fitted around each soil core after excavation. To avoid boundary effects, molten paraffin was poured around the perimeters to seal small gaps between the soil core and the pipe along the entire length. Sieved surface soil was put back on the top of the core and packed with a pressure of 460 kg/m^2 applied for 30 s. Prior to introduction of bacteria, soil columns were saturated with sterile distilled water and incubated in the growth chamber for 4 days. For bacterial application, 500 g of surface soil was inoculated with 5×10^7 CFU (each) of the wild type and a *gacA* mutant per g and incubated at 15°C for 2 days. The bacterized soil was then placed on the top of each soil column. On the same day, 19

wheat seedlings were planted and covered with a thin layer of quartz sand. Rainfall was simulated three times per week by passing 500 ml of distilled water through 19 glass capillaries (0.2-mm diameter, 10-mm length) attached to the bottom of a plastic container placed 30 cm above the microcosm tube. This corresponds to 370 mm of rainfall per 52 days. This is the maximum precipitation rate found during the last 10 years at the site of soil core extraction within such a period. Percolation water of each column was collected, and after 5 h a representative sample was used for determination of bacterial populations. Periodically, soil samples (about 1 g) from the surface layer of the column were removed. After 13 days, soil samples (about 1 g) were extracted from different soil depths through gaps in the pipe with a cork borer (diameter, 0.5 cm). At the end of the experiment, after 52 days, soil was carefully removed from the pipe and soil samples (about 5 g) were collected at different depths.

Small open microcosms. Basically, small open microcosms were constructed similarly to the large microcosms described before. However, only the 20-cm layer of sieved surface soil was placed in a standard Mitscherlich pot (20-cm internal diameter, 20-cm height). Inoculation with bacteria, planting of wheat seedlings, and rainfall simulation were carried out as described for the large open microcosms. Surface soil samples were taken prior to each rainfall event, and percolation water was collected for 3 h after rainfall simulation.

Sampling procedures and enumeration of bacteria. Populations of the introduced strains CHA0 and CHA96 at each sample date were monitored by dispersion of soil samples in sterile distilled water (by shaking at 300 rpm for 1 h and vigorously vortexing for 15 s). Suspensions were decimally diluted and plated onto S1 selective medium for fluorescent pseudomonads (21) amended with 100 μg of rifampin per ml. When mixed populations of strains CHA0 and CHA96 were used, 30 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) per ml was additionally incorporated into this medium. The detection limit was about 1 CFU/g of soil with duplicate plating, as suspensions containing 0.5 g of soil could be spread in 2 ml of H_2O onto each plate and then dried in a laminar flow cabinet. In none of the soils used in this study were interfering background populations able to grow on this medium observed. It has to be kept in mind that the values obtained by this method might underestimate the actual populations since some bacteria starving in soil cannot be cultivated on selective media (3). The indigenous populations of aerobic heterotrophic bacteria in soil were estimated on KBA, and those of resident fluorescent pseudomonads were monitored on S1 medium without antibiotics added. Bacterial populations in the percolation water were determined by decimal diluting and directly plating samples on the media mentioned above. For quantification of the rhizosphere population of introduced bacteria, intact root systems were carefully harvested. The root samples were shaken free of loose soil by gentle agitation in sterile distilled water for 5 s. Each sample was transferred to a 100-ml flask containing 50 ml of sterile distilled water, shaken for 1 h at 300 rpm, and then vigorously vortexed for 30 s. The resulting suspension was decimally diluted and plated. The roots, now without adhering soil, were saved, brought into 20 ml of sterile distilled water, and macerated in a homogenizer three times for 15 s each time. Decimal dilutions of the resulting suspension were plated and used for enumeration of bacterial populations on roots. Plates were incubated at 27°C, and colonies were counted after 2 days.

Statistics. Each experiment was repeated at different times. Means from at least three independent experiments are presented, and each experiment had three replicates per treatment. The data obtained for bacterial populations approximated a log normal distribution. Log normal distribution of bacterial rhizosphere populations has been discussed in detail by Loper et al. (36). Accordingly, means ± standard errors of the means of reported and plotted population densities were calculated with log-transformed values. When appropriate, each mean was compared with all other means by Student's *t* test, if experiments were not significantly different in an *F* test. Ratios were calculated with log-transformed values, too, and the logarithmic mean was then retransformed.

RESULTS

Persistence in autoclaved soil. Both *P. fluorescens* wild-type strain CHA0 and the *gacA* mutant CHA96 multiplied in pasteurized soil from Eschikon (Switzerland) (Table 1). Four weeks after application of $10^{6.70}$ CFU of each strain per g of soil, populations of strains CHA0 and CHA96 increased to $10^{8.06}$ and $10^{7.62}$ CFU/g of soil, respectively. Similar results were obtained in experiments in which strains were added individually (data not shown). Thus, the ability to produce antimicrobial compounds does not confer a selective advantage on strain CHA0 under sterile soil conditions, i.e., in the absence of competition with other organisms.

Persistence in nonsterile soils of different origins. In closed microcosms containing nonplanted soil kept at a constant moisture of 17%, populations of introduced strains declined exponentially (Fig. 1). The culturable cell numbers of introduced CHA0 and CHA96 remained higher in soil MS1 and in soil ME2 from the Morens region than in Eschikon soil (Fig. 1). Strain CHA0 had originally been isolated from Morens soil MS1, a disease-suppressive soil (49). Sixty days after introduction of the strains, this difference between population densities in Morens soil and Eschikon soil was significant ($P \leq 0.05$, Student's *t* test) for each sample date. In each soil, the population densities of the *gacA* mutant CHA96 were significantly below those of the wild type at every sample date (Fig. 1). Single-strain inoculation gave similar results (data not shown). It appears from these results that nonsterile soil conditions favor the wild-type CHA0 over the *gacA* mutant.

Persistence in the rhizosphere and on the roots of wheat. After application of a mixture of wild-type CHA0 and the *gacA* mutant CHA96 to wheat seedlings, the *gacA* mutant had no significant disadvantage when colonizing the rhizosphere and the roots after 21 and 42 days of growth in natural Eschikon soil (Table 2). After 21 days the root population density of the *gacA* mutant was even twice as high as that of the wild type. Single-strain inoculation experiments gave analogous results (data not shown). We infer that, at least during the observation period and in the soil studied, a *gacA* mutation does not reduce the persistence of *P. fluorescens* in the vicinity of wheat roots.

Percolation and persistence in small open microcosms. A 20-cm-deep column containing sieved surface-layer soil from Eschikon was used to introduce the wild-type CHA0 together with the *gacA* mutant CHA96. The populations in the surface soil and in the percolated water are shown in Fig. 2A. At each sample date, the ratio of the population density of the wild type to the density of the mutant was determined in surface soil and in percolated water. These ratios were always significantly ($P \leq 0.05$, Student's *t* test) higher in the surface soil. After the first rainfall simulation, 2 days after bacterial application to soil, the ratio CFU of CHA0 per CFU of CHA96 was 4.8 in the surface soil but 0.7 in the percolated water. After 52 days, this ratio was 20.0 in the surface soil and 4.2 in the percolated water. This suggests that the *gacA* mutant may have been washed out by

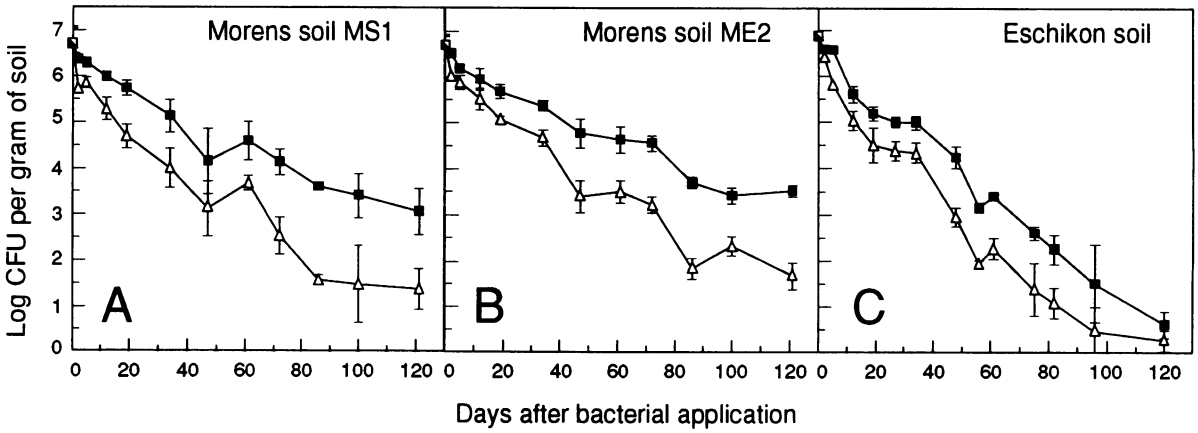

FIG. 1. Persistence of *P. fluorescens* wild-type CHA0 (■) and the *gacA* mutant CHA96 (△) in a closed system with natural, nonplanted soils of different locations kept at constant moisture (17%). (A) Soil from the original isolation site of CHA0; (B) soil from the same region; (C) soil from another region of Switzerland. Strains were established in the soil as a mixture, each strain at an initial population density of 5×10^6 CFU/g. Values are mean population densities (± standard errors) at each sample date obtained from three independent experiments with two replicates (flasks) per experiment.

the simulated rainfalls at a higher percentage than was the wild type.

In these microcosms, the resident population of aerobic bacteria was monitored on KBA and indigenous fluorescent pseudomonads were enumerated on S1 selective medium (Fig. 2B). In experiments without introduced bacteria, the resident population of aerobic bacteria as enumerated on KBA was constant at about $10^{6.9}$ CFU/g of surface soil and $10^{5.3}$ CFU/ml of percolation water. Populations of resident fluorescent pseudomonads remained stable at approximately $10^{5.1}$ CFU/g in surface soil and $10^{3.6}$ CFU/ml in percolated water (data not shown).

The experiments conducted in unplanted (Fig. 1) and planted (Fig. 2A) nonsterile soil indicated a selective disadvantage for the *gacA* mutant CHA96, relative to its parent CHA0. Since the handicap of strain CHA96 might be due to the presence of the *lacZ* insert in the *gacA* gene or even be caused by the nonisogenic nature of the rifampin resistance mutations in CHA0 and CHA96, we repeated the column percolation experiment with an independently isolated *gacA* mutant, strain CHA89 (33). This strain carries a kanamycin resistance cassette in *gacA* and was separately marked with rifampin resistance. Strain CHA89 was coinoculated with wild-type CHA0 onto a 20-cm soil column. The population densities of strain CHA89 in the surface soil and percolated water were similar to those found for CHA96, except that the final concentrations of the wild type and the mutant in the surface soil were about 0.3 log unit lower than in the experiment reported in Fig. 2A. The ratio of the wild-type population to *gacA* mutant population (CFU of CHA0 per CFU of CHA89) was also significantly higher in the surface soil than in the percolated water at every sample date (data not shown). Since the behavior of the two independently constructed *gacA* mutants was similar, it seems likely that the *gacA* mutation, rather than other differences, accounts for the reduced fitness in nonsterile soil.

Percolation, vertical distribution, and persistence in large open microcosms. Strains CHA0 and CHA96 were both introduced into the surface soil of 60-cm-long soil columns. After each of 21 simulated rainfall events, both the wild type and the mutant were detected in the water percolated through 60 cm of soil. The populations in the surface soil and in the percolated water are given in Fig. 3. Although variation in this complex system was quite high, both strains displayed overall survival

TABLE 2. Persistence of *P. fluorescens* wild-type CHA0 and the *gacA* mutant CHA96 in the rhizosphere and on the roots of wheat grown in natural Eschikon soil

Reisolation site and incubation time of introduced strains[a]	Log CFU/root system[b]		Ratio (CFU of CHA0/ CFU of CHA96)[b]
	CHA0	CHA96	
Seedling roots, before planting	6.39 ± 0.41	6.32 ± 0.61	1.19 a[c]
Rhizosphere			
21 Days after planting	4.30 ± 0.35	4.11 ± 0.36	1.64 a
42 Days after planting	3.93 ± 0.22	3.84 ± 0.21	1.23 a
Roots			
21 Days after planting	4.75 ± 0.50	5.12 ± 0.32	0.46 b
42 Days after planting	4.43 ± 0.19	4.43 ± 0.25	0.99 a

[a] For introduction of a mixture of strains CHA0 and CHA96, wheat seedlings were incubated for 15 min in a bacterial suspension containing 5×10^7 CFU of each strain per ml and then planted. Rhizosphere bacterial populations were obtained from roots with adhering soil; root populations were from thoroughly washed and macerated roots.

[b] Each value is the mean (± standard error) from five independent experiments with three replicates (pots, each with five plants) per experiment.

[c] Means with the same letter are not significantly different at $P = 0.05$ (Student's t test).

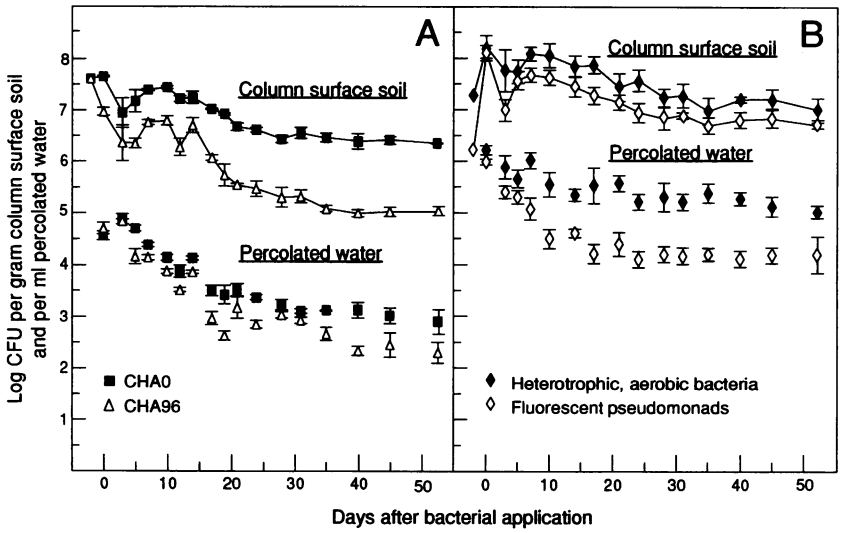

FIG. 2. Populations of *P. fluorescens* CHA0 and the *gacA* mutant CHA96 (A) and populations of aerobic bacteria isolated on King's medium B and of fluorescent pseudomonads isolated on S1 medium (B) in the surface soil and the percolated water of small open microcosms planted with wheat. A mixture of both CHA0 and CHA96 was established in 500 g of soil, each strain at an initial population of 5×10^7 CFU/g. The bacterized soil was placed on the 20-cm-long columns, consisting of sieved, packed Eschikon soil, 2 days later. Values are mean population densities (± standard errors) at each sample date from three independent experiments, each with three replicate microcosms.

and washing-out frequencies that were comparable to those in the shorter soil column (Fig. 2A). Calculated for 1 m^2, the total number of cells washed out at 60-cm depth over the entire duration of the experiment was 2.6×10^7 CFU for CHA0 and 1.5×10^7 CFU for CHA96, i.e., 0.08 and 0.05%, respectively, of the initially added inoculum.

After 13 and 50 days, the vertical distribution of the introduced bacteria in the soil columns showed a strong decrease of population densities down to a depth of 30 cm. In deeper soil layers, no further decline could be measured (Fig. 4).

The same experiments were performed with strains applied individually to each column, and similar results were obtained (data not shown).

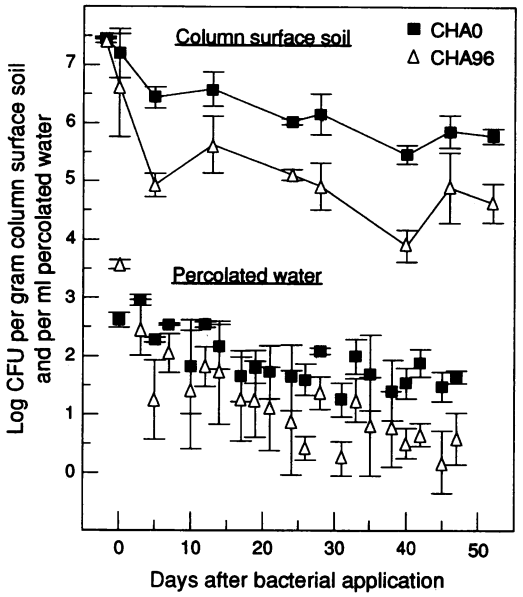

FIG. 3. Populations of *P. fluorescens* CHA0 and the *gacA* mutant CHA96 in the surface soil and percolated water of 60-cm-deep, large open microcosms planted with wheat. Microcosms consisted of soil columns (diameter, 19 cm) containing an undisturbed soil core from Eschikon. Bacterial application was as described in the legend to Fig. 2. Values are mean population densities (± standard errors) at each sample date from three independent experiments with three replicates (microcosms) per experiment.

DISCUSSION

In this study, microcosms of different complexities were used to study the influence of a *gacA* mutation on the persistence and vertical translocation of *P. fluorescens* in soil. Large microcosms with undisturbed soil cores represent natural variation and appear to be suitable for predicting the survival and percolation of introduced strains under field conditions (2, 4, 5, 18, 55). However, for monitoring differences between introduced wild-type and mutant strains, simplified systems may be equally appropriate in that they may reduce background noise. Most experiments in this study were conducted in parallel, with strains being introduced as a mixture or separately. Both methods gave similar results: the *gacA* mutants showed impaired persistence in soil. Thus, diminished survival of the mutants could not be attributed to competition with the parental strain.

Multiplication in sterile soil but exponential decrease during an initial phase after introduction of the bacteria into nonsterile soil has been reported for different *Pseudomonas* strains (1, 10, 62). Little is known about conditions under which an introduced strain can establish a stable population. Colbert et al. (9) reported that selective feeding of salicylate by drip irrigation to introduced *Pseudomonas putida* PpG7, which utilizes this carbon source because of plasmid NAH7, leads to increased population densities in agricultural soils and tomato rhizospheres. The NAH7 plasmid was transferred to a *P.*

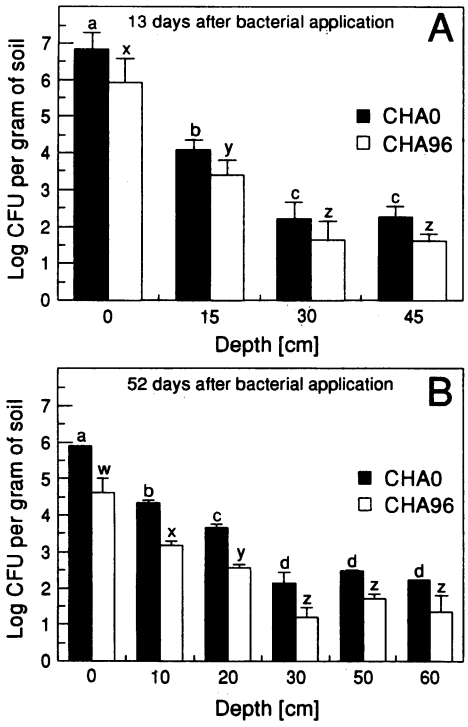

FIG. 4. Vertical distribution of populations of *P. fluorescens* wild-type CHA0 and the *gacA* mutant CHA96 13 (A) and 52 (B) days after introduction as a mixture into 60-cm-deep, large open microcosms (compare with Fig. 3). Values are the mean population densities (± standard errors) at each sample depth from three independent experiments with three replicates (microcosms) per experiment. Bars within the same panel and designated with the same letter are not significantly different at $P = 0.05$ (Student's t test).

putida biocontrol strain, resulting in similar effects under laboratory conditions (8). Selective feeding of beneficial organisms can also be achieved by the use of cover crops to increase specific populations of soil microorganisms (11). In our work, strain CHA0 survived better in the soils from the region where it had originally been isolated than in the one soil tested from another part of Switzerland. The differences in survival in soils of different origins may be due to a variety of abiotic and biotic factors known to affect persistence of introduced strains (47, 48, 54, 55).

In sterile soil, the population density of the *gacA* mutant was only slightly below that of the wild-type CHA0. This is hardly surprising, because in the absence of other competing organisms nutrient availability is thought to be the main limitation. In natural, nonsterile soil the *gacA* mutant CHA96 was less persistent; this effect was obvious soon after introduction. This suggests that the production of *gacA*-regulated extracellular metabolites and enzymes may be important for the survival of CHA0 in soil, especially during the initial phase of establishment. Several of these *gacA*-regulated metabolites, e.g., 2,4-diacetylphloroglucinol and pyoluteorin, have broad-spectrum antibacterial and antifungal activities (24, 27, 34, 39), and they may enable the introduced wild type to compete with resident microorganisms. Mazzola and coworkers (40) attributed the reduced survival of phenazine antibiotic-negative mutants in the rhizosphere of wheat and in nonsterile soil to a diminished ability to compete with the indigenous microflora, since in pasteurized soil the mutants colonized the rhizosphere to the same extent as did the parental strains. Interestingly, the impaired fitness of these phenazine-negative mutants was obvious in nonplanted soil only after several weeks (40). After application to seedlings the *gacA* mutant was not less persistent than was the wild type in the rhizosphere and on the roots of wheat grown in Eschikon soil. The fact that strain CHA96 showed better survival on wheat roots than in soil does not seem to be a plant-specific phenomenon, since in parallel experiments no significant differences were observed between populations of the wild type and the mutant on the roots and in the rhizosphere of cucumber (our unpublished data). In other experiments involving root or seed inoculation, survival of *Pseudomonas* strains in the root environment was not affected by antibiotic-negative mutations in the course of 10 to 14 days (25, 32, 52). In contrast, Mazzola et al. (40) found that after application of the bacteria to the soil, introduced phenazine-negative strains were significantly less persistent than the parental strains, not only in soil but also in the rhizosphere. Several antimicrobial metabolites relevant for disease suppression have been shown to be produced in the rhizosphere (27, 53, 61); whether they are also produced in unplanted soil has not been determined. Since the *gacA* gene regulates antimicrobial metabolites and some exoenzymes (43), we cannot be certain that all ecological effects caused by a *gacA* mutation are due to the loss of antibiotic production only.

Percolation of introduced CHA0 and its derivatives was monitored in soil microcosms in a situation that simulates repeated short but heavy rainfall events. The concentration of introduced bacteria in percolated water was highest in the beginning, but possible translocation of introduced bacteria to soil layers below 60 cm has been shown to happen under these conditions constantly over a longer period. In analogous experiments, Fredrickson et al. (18) detected *Pseudomonas* Tn*5* mutants, after inoculation of a 15-cm-deep layer of topsoil, in the percolation water of 60-cm-long columns at a concentration of about 10^3 CFU/ml after 21 days. van Elsas et al. (55) conducted short-term experiments in 50-cm-long, undisturbed columns with an inoculum in the surface soil similar to that used in our experiments. They found that an introduced Tn*5*-tagged *P. fluorescens* strain could be recovered at a concentration of about 10^2 CFU/ml in the percolation water during 2 days. This value is below that found in our experiments, in which the initial concentration was between 10^3 and 10^4 CFU/ml. In our studies, the vertical distribution of the bacteria in large soil columns showed a strong population decline in the upper layers consisting of repacked soil and no significant decrease within the undisturbed core. A comparison of the bacterial percolation in the 20-cm-long columns consisting of sieved soil and the 60-cm-long columns containing undisturbed soil cores supports the finding that the undisturbed soil core did not retain these bacteria very efficiently: cell numbers in the percolation water at 60 cm were still about 5% of those found at 20 cm. Similarly, van Elsas et al. (55) found higher percolation in undisturbed soil cores than in repacked soil cores. This could be due to preferential flow, which means that substances are not translocated uniformly through soil but preferentially follow macropores, cracks, earthworm channels, and root channels, etc. (19).

In this study, the *gacA* mutants showed percolation properties different from those of the wild-type CHA0. Bacterial counts in percolation water are influenced by survival and translocation properties of a strain. In short and long soil columns the numbers of *gacA*-negative cells in the percolated water were smaller than those of the wild type. Nevertheless, the increased percolation of the mutant from the short column was highly significant; this was evident from the following

finding. The mutant survived poorly in topsoil, compared to the wild type. In percolation water from the short soil columns, however, the wild type and the mutant gave similar cell counts. In the large soil columns the same tendency was found, but variation was quite high and the population densities in the surface soil were not determined often enough to prove a significant effect. Previously, de Flaun et al. (15) characterized adhesion-deficient mutants of *P. fluorescens*, which are washed out of soil more readily. Smit et al. (45) suggested that different percolation properties of a mutant might be a risk. Our results show that in fact a mutation in a global regulator gene may change percolation properties. This side effect of the *gacA* mutation should be considered in risk assessment as an example of an unpredicted ecological behavior of an introduced strain.

In conclusion, the results of this study and work by others (40) suggest that the production of metabolites which are important for disease suppression in biocontrol pseudomonads may also contribute to these strains' ecological competence. A single mutation in a global regulator gene was also shown to have complex consequences on the behavior of *Pseudomonas* strain CHA0 in soil ecosystems.

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